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Isolation of novel *Pseudomonas syringae* promoters and functional characterization in polyhydroxyalkanoate-producing pseudomonads

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A library of genomic DNA fragments of *Pseudomonas syringae* pv. *tomato* DC3000 was constructed in a *lacZ*α-containing plasmid, pBS29. The library was used in a preliminary α-complementation-based screen to identify clones with promoter activity in *Escherichia coli*. Ten positive clones were sequenced and their locations in the chromosomal DNA of DC3000 strain were mapped. Five positive clones (P2, P3, P4, P6 and P8) were further assayed for promoter activity in three polyhydroxyalkanoate-producing pseudomonads: *Pseudomonas resinovorans*, *P. corrugata* and *P. chlororaphis*. To this end, a green-fluorescent-protein gene (*gfp*) was cloned downstream from the putative (DC3000) promoter in a shuttle plasmid. We found that only *Pseudomonas* transformants harboring the *gfp*-containing plasmid driven by putative promoter P2 showed fluorescence, indicating that this promoter is functioning in the three tested pseudomonads. Results of an *in silico* analysis of the P2 sequence further support the assignment of P2 as a bona fide promoter by the localization of putative −10 and −35 promoter regions and a transcription-factor-binding site, rpoD17, in this sequence. We successfully applied promoter P2 to drive the expression in *P. chlororaphis* of a recombinant α-galactosidase gene of *Streptomyces coelicolor*, which should be useful for the utilization of oligosaccharides of soy molasses for the production of polyhydroxyalkanoate biopolymer or rhamnolipid biosurfactant.

Introduction

Pseudomonads are a genus of bacteria that have much economic, environmental and health significance. Many of these bacteria are plant or opportunistic human pathogens that on one hand cause irreparable economic and social losses but can nevertheless also be beneficially used as selective biocontrol agents. Other pseudomonads have versatile metabolic capacity to degrade organic compounds including some environmentally detrimental or notoriously recalcitrant industrial contaminants; these have been advantageously employed in bioremediation undertakings. Still other pseudomonads are producers of useful compounds with proven or potential industrial applications. With such a vast expanse of areas of importance, it is imperative that molecular engineering tools are widely available to modulate the metabolic capacities of this group of bacteria to better benefit our needs.

Expression plasmid vectors containing an appropriate promoter are required for the expression of a gene(s) of interest in the target organism during a molecular engineering event. With a few exceptions, the strong P_{tac} and other similar promoters originating from *Escherichia coli* are commonly used in the construction of expression vectors for pseudomonads [1–4]. On one hand, it is fortunate that the *E. coli* promoters function well in pseudomonads and no extensive research is required to find additional promoters for use in this group of bacteria. However, the lack of efforts to characterize *Pseudomonas* promoters could lead to the omission of *cis*-acting transcriptional activation elements unique to the gene regulation pattern of pseudomonads. Furthermore, gene expression in

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Pseudomonas might not be optimal when the commonly used *E. coli* promoters are used [5]. In this paper, we present our study on the identification and functional characterization of chromosomal DNA sequences containing promoter activity isolated from *Pseudomonas syringae* pv. *tomato* DC3000 (hereon referred simply as DC3000). These sequences were first identified by screening an existing promoter trap library [6] for activity in *E. coli*. We further demonstrated using green-fluorescent-protein gene as a model that one of the isolated promoters, P2, functions well in *P. resinovorans*, *P. corrugata* and *P. chlororaphis*. These three *Pseudomonas* species are tested because of their capability to synthesize medium-chain-length polyhydroxyalkanoates (mcl-PHA), a class of important biodegradable polymers [7–9], under unique conditions. For example, *P. resinovorans* is capable of using intact triacylglycerols (i.e. animal fats and vegetable oils) as carbon sources to synthesize mcl-PHA [10]. *P. corrugata* produces mcl-PHA with high molecular weight even at growth temperatures higher than 30°C [11]. The possibility of coproducing other valorised products along with mcl-PHA adds to the attractiveness of using *P. corrugata* as a producing strain [12]. The versatile capability of *P. chlororaphis* to produce both mcl-PHA and the rhamnolipid biosurfactant [13] renders this organism particularly attractive for the development of industrial bioprocesses. We proceeded to demonstrate that promoter P2 is useful for the expression of a recombinant α-galactosidase gene of *Streptomyces coelicolor* in *P. chlororaphis*.

Materials and methods

Bacteria, growth conditions and plasmids

E. coli DH5α used in routine DNA subcloning and plasmid maintenance was purchased from Invitrogen (Carlsbad, CA, USA). *Pseudomonas corrugata* 388 was obtained from the late Dr. W.F. Fett (Eastern Regional Research Center/ARS/USDA, Wyndmoor, PA, USA); the strain was originally isolated from alfalfa roots by F.L. Lukezic (Pennsylvania State University, University Park, PA, USA). *P. chlororaphis* NRRL B-30761 and *P. resinovorans* NRRL B-2649 were obtained from the ARS Culture Collection (NCAUR, Peoria, IL, USA). Plasmid pET28-dAG [14] consisted of the gene coding for the N-terminal catalytic domain of a *S. coelicolor* α-galactosidase cloned into the expression vector pET28, was a kind gift of Prof. H. Kobayashi (Koibuchi College of Agriculture and Nutrition, Koibuchi, Japan). Bacterial strains were maintained on Luria medium (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl) or tryptic soy broth (TSB; Difco, Detroit, MI). Growth of *E. coli* was performed at 37°C and the *Pseudomonas* at either 30 or 37°C as specified. Agar media were prepared by including 1.2–1.5% w/v of agar to the corresponding liquid broths before autoclaving. Kanamycin (Km, 35 μg/ml), tetracycline (Tc, 12 μg/ml) and carbenicillin (Cb, 50 μg/ml) were added to the growth media as needed.

Molecular cloning procedures

Restriction digestion, 5′-dephosphorylation and DNA ligation reactions were performed using the appropriate restriction enzyme(s), calf intestinal alkaline phosphatase and T4 DNA ligase obtained from common commercial sources according to the supplier's instructions. Polymerase chain reaction (PCR) was performed using commercially available thermotolerant DNA polymerase. For the PCR-cloning of a functional gene (i.e. the truncated α-galactosidase or dAG), the high-fidelity PrimeSTAR HS DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) was employed. PCRs were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using thermal cycling programs appropriate for the specific primers used. Isolation of DNA restriction fragments or PCR products from agarose gel was carried out using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). Transformation of *Pseudomonas* strains by electroporation was performed according to a previously described protocol [15]. Plasmids in *E. coli* transformants were isolated by using a GenElute Miniplasmid Kit (Sigma, St. Louis, MO, USA) for subsequent restriction analysis. A slightly modified alkaline lysis method [16] was used to isolate plasmids from *Pseudomonas* transformants. Briefly, cells from overnight culture (3–5 ml) were collected by centrifugation and washed twice by sequential resuspension and centrifugation in 500 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.9). The washed cell pellet was resuspended in 100 μl TE buffer, and 20 μl of a 10 mg/ml lysozyme solution was added. The mixture was incubated at 37°C for 15 min. Upon cooling to ambient room temperature, the lysozyme-treated cell suspension was processed through the alkaline-SDS lysis protocol as described [16]. Plasmid DNA in the cleared cell lysate was precipitated by adding 2.5× volume of chilled ethanol and incubated in a freezer (−20°C) for at least 16 hours. The DNA was collected by centrifugation, washed once with 70% ethanol at ambient room temperature, dried by evaporation in a vacuum centrifuge (rotovac) and resuspended in an aliquot (20–50 μl) of deionized water.

Comparative measurement of green-fluorescent-protein (*gfp*) expression

Pseudomonas transformants were cultured for 36–48 hours at 30°C and 200–250 rpm shaking in 3.5 ml of medium E* [17] supplemented with glucose (2% w/v; Sigma Chemicals) and an appropriate antibiotic as a selection pressure. The parental wild-type strains used as control samples were similarly cultured without the addition of antibiotic. At harvest, the cultures were centrifuged to obtain the supernatant and cell pellet fractions. The cell pellets were resuspended in 2 ml of deionized water. In a separate experiment with *P. resinovorans*, we further confirm the presence of GFP in the supernatant fraction. Accordingly, the supernatant was filtered through a 0.2-μm PVDF membrane housed in 13-mm Acrodisc LC syringer-filter (PALL Life Sciences, Port Washington, NY) to remove any residual bacteria and the fluorescence of the filtrate was measured. This filtrate was then subjected to another filtration using an Amicon Ultra 10K centrifugal filter device having a 10,000 Nominal Molecular Weight Limit (Millipore, Billerica, MA). The fluorescence of the ultrafiltrate was then measured. The retained species was analyzed by denaturing discontinuous gel electrophoresis on a Mini PROTEAN 3 Cell apparatus (BioRad Laboratories, Hercules, CA), using a Ready Gel Precast 12% polyacrylamide gel (BioRad Laboratories). An equal volume of the retained species was mixed with a 2× Sample Loading Buffer solution [18] and heated to 95°C for 4 min. Upon cooling to room temperature, an aliquot of the sample was loaded on the polyacrylamide gel, and electrophoresis was performed in Tris-glycine–SDS buffer system [19]. Proteins in the gel were visualized using a SimplyBlue™ SafeStain kit (Invitrogen) according to the manufacturer's instructions. Relative fluorescence of all samples was recorded at $\lambda_{\text{emission}}$ of 525–527 nm, with the $\lambda_{\text{excitation}}$ set at 481–485 nm. Front fluorescence mode was used to measure the relative fluorescence of the cell suspension. Two clones from each strain or transformant were used in the experiment, and two separate cultures were set up for each clone. Three fluorescence readings were averaged and recorded for each sample. With rare exceptions, we noted that the standard errors for the averages of these fluorescence measurements were less than 1% for each sample.

Assay of α-galactosidase activity

P. chlororaphis transformants were cultured for two days (at 30°C and 200 rpm) in LB + Tc medium (50 ml) in 125-ml Erlenmeyer flasks. For each culture, cells were harvested by centrifugation (Sorvall RC-5B centrifuge, SS-34 rotor, 6500 rpm, 15 min, 4°C) and washed once with 5 ml of HEPES buffer (0.1 M, pH 7). Cell pellet was resuspended in 1 ml of HEPES buffer in a 15-ml COREX centrifuge tube, and the resultant cell suspension was subjected to sonication (Model W-385 Sonicator with the Output Control set at 5; Heat Systems-Ultrasonics, Inc., Farmingdale, NY; fitted with a standard tapered microtip) to disrupt the cells. Each sample was sonicated for 5 × 10 s with cooling in ice-water between sonication. Cell extract was obtained by centrifugation (Sorvall RC-5 centrifuge, SS-34 rotor, 9000 rpm, 30 min, 4°C) of the sonicated cell suspension. Alpha-galactosidase activity of the cell extract was assayed essentially as described by Kondoh *et al.* [14]. Briefly, 0.1 ml of cell extract was added to 0.9 ml of 1 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*-NP-α-gal) in 0.1 M HEPES (pH 7) in a test tube. After a 20-min incubation at 40°C, 1 ml of 0.2 M Na_2CO_3 solution was added to stop the reaction. The concentration of *p*-nitrophenol (*p*-NP) released by α-galactosidase enzyme activity was determined based on the absorbance at 400 nm of the reaction mixture and the extinction coefficient of *p*-NP of $\varepsilon = 17{,}500\ \text{M}^{-1}\ \text{cm}^{-1}$ [20]. Protein concentration of the cell extract was determined by using a commercial kit (Protein Assay Dye Reagent Concentrate Kit, BioRad Laboratories, Hercules, CA) and bovine serum albumin as a reference. We define one unit of enzyme activity as the amount of enzyme that catalyzes the release of 1 nmole of *p*-NP per 20 min under the reaction conditions.

Results and discussion

Isolation of DC3000 promoters with constitutive activity in E. coli

We employed an unbiased approach to identify promoters that could be used to drive high-level constitutive expression of target genes in PHA-synthesizing pseudomonads as well as in *E. coli*. This strategy used a conventional promoter trap vector, which contains a library of DC3000 genomic DNA fragments cloned upstream of a promoterless *lacZα* gene. A complete description of the promoter trap vector and library construction has been reported previously [6]. Briefly, the promoter trap vector (pBS29) was designed to detect transcriptional activity originating from DNA sequences cloned upstream of the *lacZα* reporter gene. This vector was designed as a transcriptional fusion and as such, upstream of the *lacZα* gene were stop codons for all three reading frames, followed by a ribosome binding site (RBS). This 5′–3′ stop codons-RBS-*lacZα* configuration was designed to minimize differences of translation efficiency on reporter expression, and instead focus the analysis on transcription. The promoter trap genomic library used in these experiments was constructed by cloning DC3000 genomic DNA fragmented by partial digestion with *Sau*3AI into the *Bam*HI site directly upstream of the three stop codons. The resultant library would thus express the *lacZα* reporter gene only when an active promoter is cloned upstream of the reporter gene. The screen was carried out in *E. coli* cells that were transformed with the library, and clones containing constitutively active promoters were identified by the ability to produce a blue color on 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) indicator media. *E. coli* was used in this first-line screening because of the versatility of this genetic-engineering 'workhorse' for a rapid and convenient genetic manipulation to identify isolates that contain potential promoters. An added advantage of using *E. coli* initially as a promoter-screening host organism is that the eventually isolated *Pseudomonas* promoter sequences would be functional in both *E. coli* and *Pseudomonas*, rendering these broad-host-range promoters even more useful for industrial bioprocess applications. Using this approach, a total of 3879 transformants were screened and of these approximately 17% displayed the *lac+* phenotype that was indicative of an active promoter. Ten clones were chosen at random from the *lac+* transformants for further analysis based on the production of an intense blue color on the assay plates.

TABLE 1

Genomic coordinate[a] and size of (DC3000) promoter-containing library inserts

Promoter clone	5′	3′	Size (bp)
1a	4306996	4306263	790
1b	4004433	4005223	733
2	6143993	6144829	837
3	5762792	5763679	887
4	3721663	3720696	907
5a	1966180	1967068	671
5b	393137	392466	888
6	6347364	6346425	939
7	5014461	5015362	901
8	4069140	4068212	928
9	4069140	4068212	928
10	2680408	2681447	1039

[a] The nucleotide coordinate as defined in GenBank Accession Number AE016853.

The presence of the insert was confirmed by PCR and the sequence of the insert was determined. After the sequence was determined the position in the DC3000 genome where the cloned insert was derived from was located using BLAST analysis [21]. The results in Table 1 show that single inserts were observed in eight out of the ten clones, and that the other two clones contained chimeras that were composed of fragments from two unlinked regions of the genome. The location of inserts mapped to diverse regions of the DC3000 genome and included both intergenic and intragenic regions (Table 1). All eight promoters (i.e. P2–P4, and P6–P10) containing only single inserts were selected for further investigation to evaluate promoter activity in *Pseudomonas* species.

*Construction of **gfp**-expression plasmids*

The eight DC3000 promoters investigated further in this study were originally shotgun cloned into a pBS29 vector as described in the preceding section. This family of promoter-containing plasmids is labeled pBS29-P*x*, where *x* indicates the identity of the promoter (Fig. 1). In the original study to isolate these promoters, the *lacZα* gene sequence immediately downstream from the promoter served as a reporter in an α-complementation screening with the appropriate *E. coli* host containing a truncated β-galactosidase gene [22]. This approach cannot be used for the evaluation of the transcriptional initiation activity of these promoters in the three PHA-producing *Pseudomonas* strains (i.e. *P. resinovorans*, *P. corrugata* and *P. chlororaphis*) because these bacteria do not possess the necessary genes for *lacZα*-complementation. We therefore elected to use a green-fluorescent-protein gene (*gfp*) as a reporter. A similar gene was shown to function in many *Pseudomonas* species (see e.g. [1]). The *gfp* gene used in this study was amplified from a pBS12 plasmid (Fig. 1). We designed PCR primers, CL12-119FA (5′-AAACGACGGCCAGTGCCAAG-3′) and CL12-119RA (5′-CCAATAAGCTTCTTCCGGCTCGTATGTTG-3′), to amplify the *gfp* gene from pBS12. A *Hin*dIII recognition sequence (underlined) was built into the CL12-119RA primer, and an intrinsic

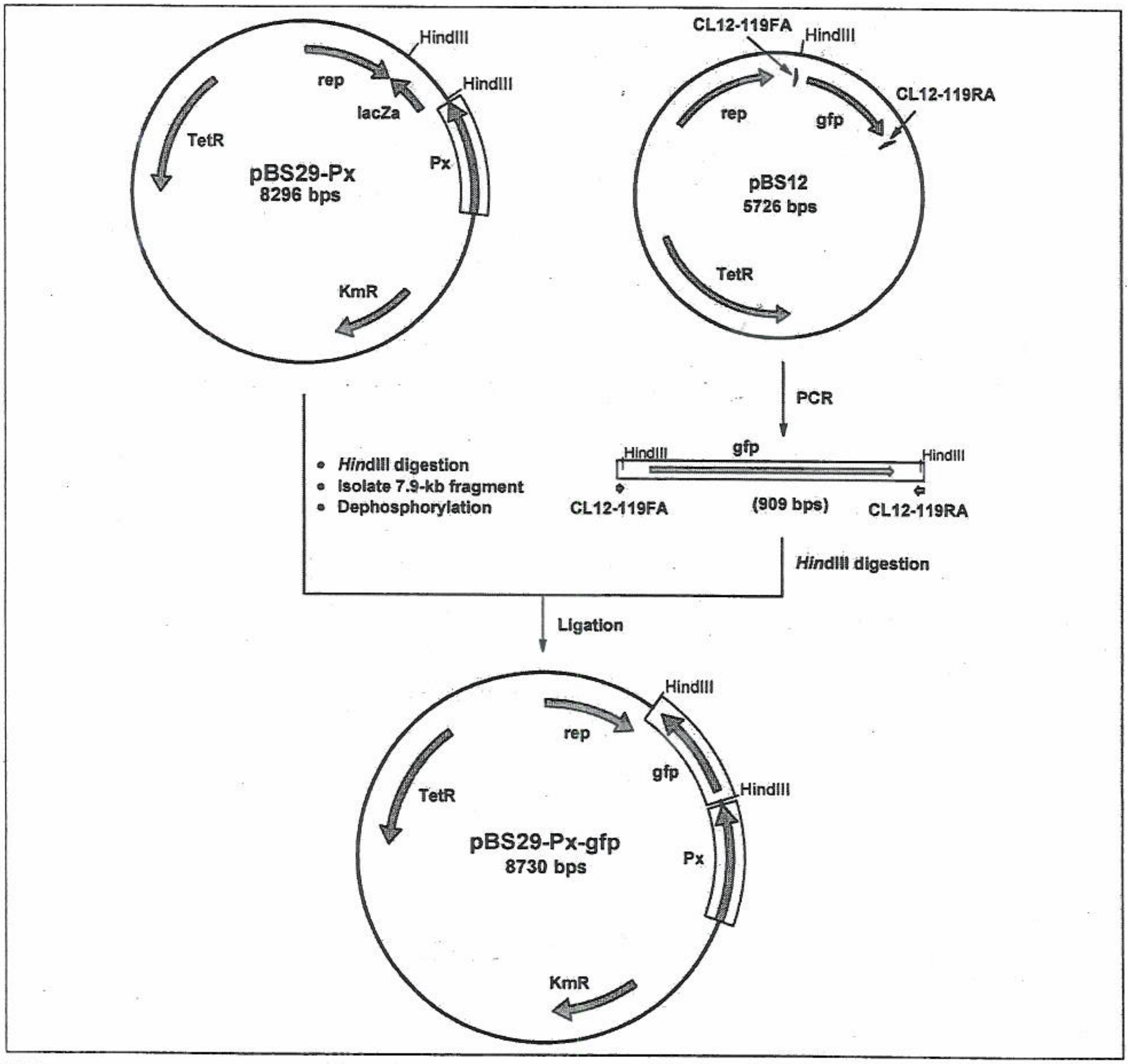
HindIII
rep
HindIII
lacZa
TetR
Px
pBS29-Px
8296 bps
KmR
CL12-119FA
HindIII
CL12-119RA
rep
gfp
pBS12
5726 bps
TetR
PCR
HindIII
gfp
HindIII
CL12-119FA
(909 bps)
CL12-119RA
• HindIII digestion
• Isolate 7.9-kb fragment
• Dephosphorylation
HindIII digestion
Ligation
HindIII
rep
gfp
HindIII
TetR
pBS29-Px-gfp
8730 bps
Px
KmR

FIGURE 1

Schematic diagram depicting the construction of pBS29-Px-*gfp* plasmids. *Px*, promoter designate, where *x* = 2, 3, 4 and 6; *TetR* and *KmR*, tetracycline- and kanamycin-resistance determinants, respectively; *lacZα*, alpha-complementation fragment of *lacZ* gene; *rep*, gene coding for a replication protein; *gfp*, green fluorescent protein; *CL12-119FA* and *CL12-119RA*, binding sites of PCR primers used in the amplification of *gfp* gene. Plasmids were not drawn to exact proportion.

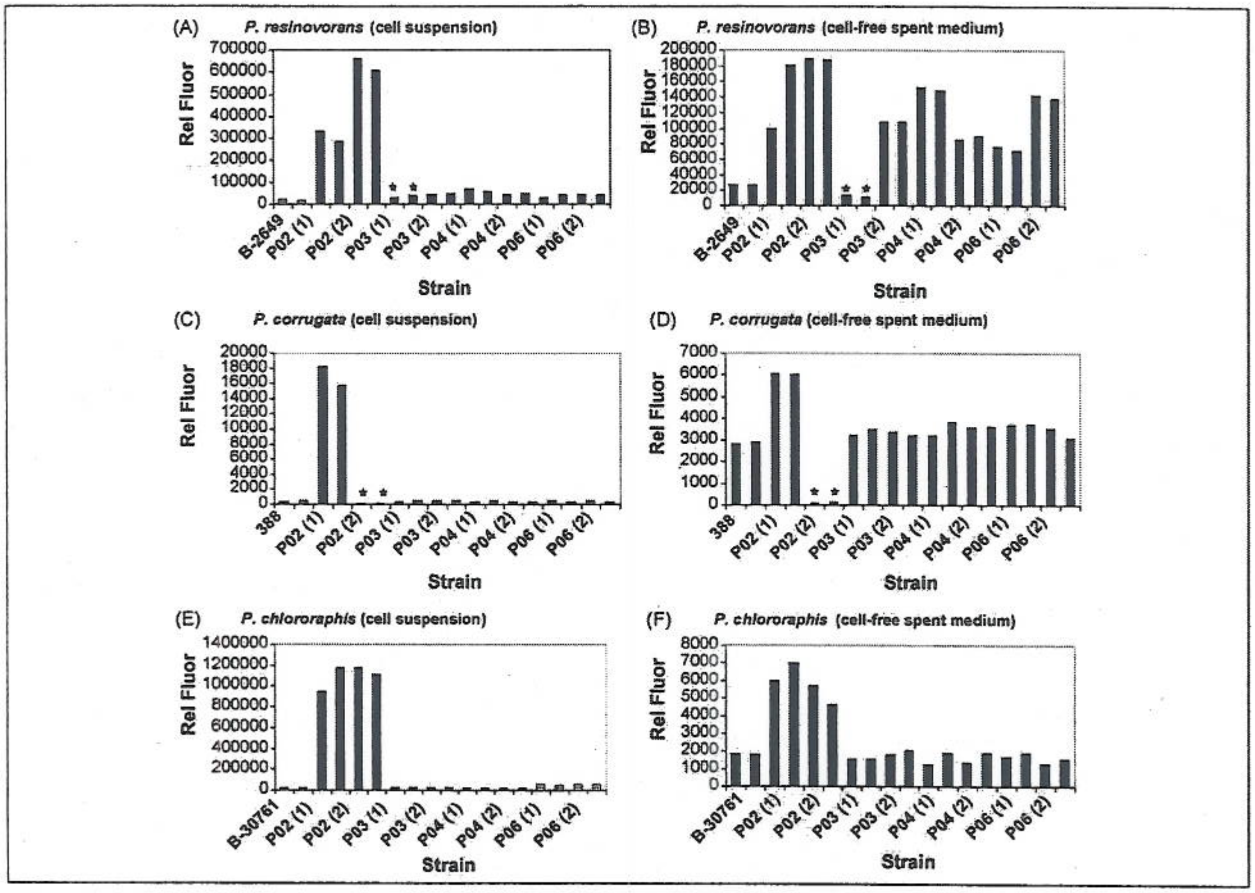

FIGURE 2

Relative fluorescence measurement of *Pseudomonas* cultures. Cultures were grown for 46 hours (*P. resinovorans*) or 48 hours (*P. corrugata* and *P. chlororaphis*) as described in 'Materials and methods'. Cell pellet and cell-free spent medium were obtained by centrifugation. The cell pellets were resuspended in deionized water. The relative fluorescence of the resuspended cell samples [(a), (c) and (e)] and the cell-free spent media [(b), (d) and (f)] were recorded at $\lambda_{emission}$ = 525–527 nm with $\lambda_{excitation}$ set at 481–485 nm. Asterisks (★) represent that the cultures did not grow or grew only poorly.

*Hin*dIII site occurs immediately following the CL12-119FA primer binding site. The amplified *gfp* (0.91 kilobase-pair or kb) was digested with *Hin*dIII restriction enzyme, and was used to replace the *Hin*dIII-flanked *lacZα* sequence of pBS29-P*x* to obtain the pBS29-P*x*-*gfp* recombinants. To do this, the individual pBS29-P*x* plasmid was digested with *Hin*dIII. The large DNA fragment (7.9 kb) was isolated by gel elution, dephosphorylated with calf intestinal alkaline phosphatase (to prevent self-ligation) and then ligated with the PCR-amplified 0.91-kb *gfp* gene. The recombinant plasmids were used to transform *E. coli*, and miniprep plasmid screening coupled with restriction analysis using an appropriate enzyme(s) allowed for the identification of plasmid constructs with the *gfp* properly oriented for expression by the *P. syringae* promoter. These plasmids are labeled pBS29-P*x*-*gfp* where *x* is the promoter number. We succeeded in obtaining pBS29-P*x*-*gfp* for P2, P3, P4, P6 and P8, but for an unknown reason were unable to isolate similar plasmid constructs containing P7, P9 and P10 through repeated attempts.

Expression of green-fluorescent-protein in **Pseudomonas**

The ability of the DC3000 promoters to drive gene expression in *P. resinovorans*, *P. corrugata* and *P. chlororaphis* was assessed. The pBS29-P*x*-*gfp* plasmids were individually introduced into the test organisms by electroporation. Two clones (or independent isolates) from each transformed bacterium were cultured for 46–48 hours, and the relative fluorescence intensity of the cell-free spent culture medium and the resuspended cell pellet were measured. Figure 2a,b shows the results of the experiment performed with *P. resinovorans* parental and transformed cells. In Fig. 2a, only the cell suspension samples of the two *P. resinovorans* clones containing pBS29-P2-*gfp* plasmid (P02 (1) and P02 (2)) show a significantly higher relative fluorescence in comparison to the wild-type strain and the other transformants harboring P3, P4 or P6 promoter. The relative fluorescence measurement of the cell-free spent medium from the *P. resinovorans* cultures unfortunately had considerable variability even between clones of a same transformant. For example, while the two cultures of clone #1 of *P. resinorovans* [pBS29-P4-*gfp*] transformant (Fig. 2b, P04 (1)) had a relative fluorescence of ~150,000 (arbitrary scale), clone #2 of the same transformant (Fig. 2b, P04 (2)) showed a significantly lower relative fluorescence of ~90,000. Marked variation between clones of a same transformant or even between the duplicate cultures of a same clone (i.e. Fig. 2b, the two P02 (1) samples of separate *P. resinovorans* [pBS29-

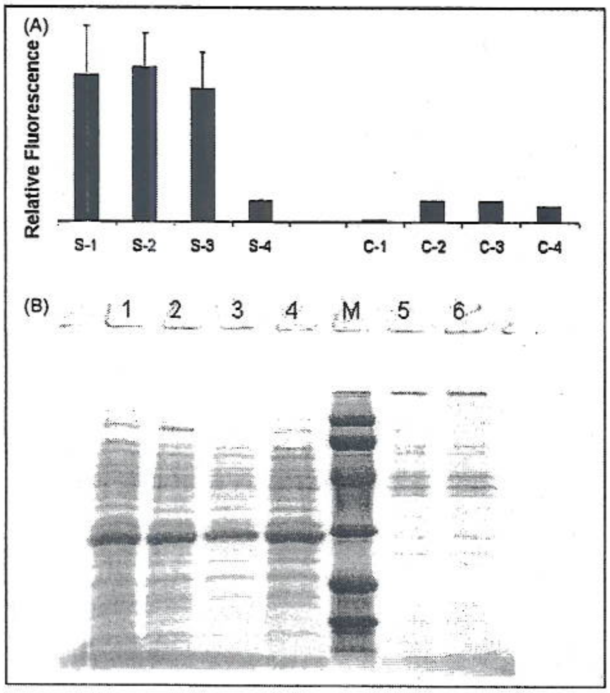

FIGURE 3

Verification of GFP in *P. resinovorans* [pBS29-P2-*gfp*] spent medium. Two clones of *P. resinovorans* [pBS29-P2-*gfp*] transformant and one clone of parental strain NRRL B-2649 were cultured in duplicate in E* medium for a total of six cultures. As described in 'Materials and methods', cells were harvested at 48 hours of cultivation and resuspended in deionized water. The supernatant fractions were sequentially filtered through membrane filters. The retained materials obtained from the final filtration step using a 10,000 Nominal Molecular Weight Limit (NMWL) membrane were subjected to denaturing discontinuous polyacrylamide gel electrophoresis on a 12% gel. **(a)** The average of four (for each of S-1 to S-4) and two (for each of C-1 to C-4) fluorescence readings from various samples obtained with *P. resinovorans* [pBS29-P2-*gfp*] and the parental strain, respectively. S-1 and C-1, cell suspensions of transformant and parental strains, respectively; S-2 and C-2, supernatant of cell-free spent medium; S-3 and C-3, 0.2-μm filtrates of supernatant samples; S-4 and C-4, 10,000 NMWL filtrates of the 0.2-μm filtrates. The error bars represent the standard deviations of the measurements. **(b)** Gel electrophoresis of 10,000 NMWL retantates. 1–4, samples from the four cultures of *P. resinovorans* [pBS29-P2-*gfp*]; *M*, low-range SDS-PAGE molecular weight standards (BioRad Laboratories) with markers of (from top to bottom) 97.4, 66.2, 45.0, 31,0, 21.5 and 14.4 kDa; 5–6, samples from the two cultures of parental *P. resinovorans* NRRL B-2648 strain.

P2-*gfp*] cultures) renders the data from the cell-free spent medium samples of *P. resinovorans* cultures difficult to interpret. To ascertain that the fluorescence in the supernatant of *P. resinovorans* [pBS29-P2-*gfp*] cultures was because of GFP, we carried out a series of filtration experiments, followed by a gel-electrophoretic analysis. Figure 3a shows the results of fluorescence measurement of various samples. The cell suspension (S-1 in Fig. 3a), supernatant of culture medium (S-2) and the filtrate from the 0.2 μm-membrane-filtration (S-3) of *P. resinovorans* [pBS29-P2-*gfp*] transformant exhibited a high level of relative fluorescence. After filtration through the 10,000 Nominal Molecular Weight Limit (NMWL) ultrafiltration device; however, the fluorescence of the filtrate of *P. resinovorans* [pBS29-P2-*gfp*] (S-4 in Fig. 3a) was dramatically reduced to the background value similar to those exhibited by the control samples from the wild-type *P. resinovorans* NRLL B-2648 (C-1, C-2, C-3 and C-4 in Fig. 3a). These results indicated that the molecular weight of the fluorescence species is higher than 10 kDa. The results of the gel-electrophoretic experiment (Fig. 3b) further showed that the fluorescence species in the supernatant of *P. resinovorans* [pBS29-P2-*gfp*] culture, which was retained in the 10,000 NMWL ultrafiltration device, had a molecular weight of 27 kDa. This value corresponds well to the molecular weight of the 27-kDa GFP [23]. The results further showed that the supernatant of the wild-type *P. resinovorans* NRRL B-2648 culture, which exhibited only a background-level fluorescence reading, did not contain the 27 kDa protein, supporting the hypothesis that the fluorescence observed in the transformant was because of GFP. The presence of GFP in the culture medium of the *P. resinovorans* transformant is unlikely to be because of protein secretion, because the construction of the promoter trap plasmid had precluded a translational read-through from the cloned putative promoter sequence. The probable explanation is that in the stationary phase, cell lysis of aged bacteria had occurred, causing the release of GFP into the culture medium. Irrespective of the reason for the presence of GFP in the culture medium, we can definitively conclude based on the collective data obtained to this point that the DC3000 promoter P2 is highly active in *P. resinovorans* as evidenced by the expression of *gfp* in the organism.

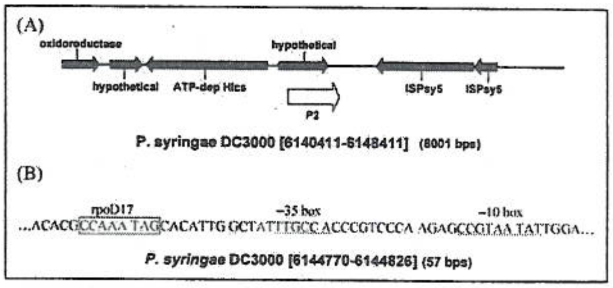
(A)
oxidoreductase
hypothetical
hypothetical
ATP-dep Hlcs
P2
ISPsy5
ISPsy5
P. syringae DC3000 [6140411-6148411] (8001 bps)
(B)
rpoD17
−35 box
−10 box
...ACACGCCAAA TAGCACATTG GCTATTTGCC ACCCGTCCCA AGAGCCGTAA TATTGGA...
P. syringae DC3000 [6144770-6144826] (57 bps)

FIGURE 4

Genomic location and characteristics of (DC3000) P2 promoter. **(a)** A schematic drawing depicting the putative genes annotated in the vicinity (between chromosome coordinate 6140411 and 6148411) of the P2 promoter. *Oxidoreductase*, a putative molybdopterin-binding oxidoreductase; *hypothetical*, hypothetical proteins; *ATP-dep Hlcs*, a putative ATP-dependent DNA helicase domain protein; *ISPsy5*, sequences related to putative transposase and Orf1 of insertional sequences. **(b)** Details of a region in P2 sequence showing the −10 and −35 promoter regions and the transcription-factor-binding site, rpoD17.

Figure 2c–f further present the results obtained with *P. corrugata* and *P. chlororaphis*. Unlike the experiment with *P. resinovorans*, the relative fluorescence readings of all samples (i.e. cell suspension and cell-free spent medium) are consistently reproducible. (The only exception was the lack of fluorescence exhibited by the samples from the two cultures of clone #2 of *P. corrugata* [pBS29-P2-*gfp*] transformant (i.e. samples P02 (2) in Fig. 2c,d), which was because of the failure of the cultures to grow for an unknown reason.) Similar studies performed with the pBS29-P8-*gfp*-transformed *P. resinovorans*, *P. corrugata* and *P. chlororaphis* cells showed that promoter P8 did not drive the expression of the heterologous *gfp* gene in these *Pseudomonas* species (data not shown). The results collectively confirmed that DC3000 promoter P2 successfully drove the expression of *gfp* in the three *Pseudomonas* species tested. The relative strength of the promoter in the three tested organisms cannot be determined from the results of these experiments.

Sequence analysis of DC3000 promoter P2

We decided to analyze the sequence of DC3000 promoter P2 because of its capacity to function in the three tested *Pseudomonas* species. The chromosomal genomic DNA sequence with its annotated features in the vicinity of the P2 promoter was retrieved from GenBank Accession Number AE016853 [24] and is shown in Fig. 4a. The P2 promoter is located upstream of, but in the opposite direction to, a coding sequence putatively identified as an ATP-dependent DNA helicase domain protein (ATP-dep. Hlcs). The P2 sequence is in fact located inside a putative hypothetical coding sequence (Fig. 4a). We next subjected the sequence in pBS29-P2-*gfp* that includes the P2 promoter and the coding region of the *gfp* gene to a promoter and transcription factor (TF) analysis using a BPROM program (www.softberry.com, Mount Kisco, NY, USA). The results of the *in silico* analysis show that putative promoter signals (i.e. the −10 and −35 regions) are identifiable in the P2 sequence (Fig. 4b). A close examination of the sequence of the putative −10 region of P2 showed that it is a composite of the consensus −10 sequences of *E. coli* or related bacterial promoters recognized by RNA polymerase containing sigma-70 (σ^{70}) or σ^{32} factor [25]. Similarly, the putative −35-region sequence of P2 matches that of the *E. coli* σ^{70} promoter [25]. The σ^{70} and σ^{32} factors are responsible for the transcription activation of housekeeping and heat-shock genes, respectively. Figure 4b shows that the *in silico* analysis also identified in the P2 sequence a putative TF binding site belonging to the rpoD17 class [26]. RpoD17 binding sites are a subclass of σ^{70} promoters, in which a 17-nucleotide spacer

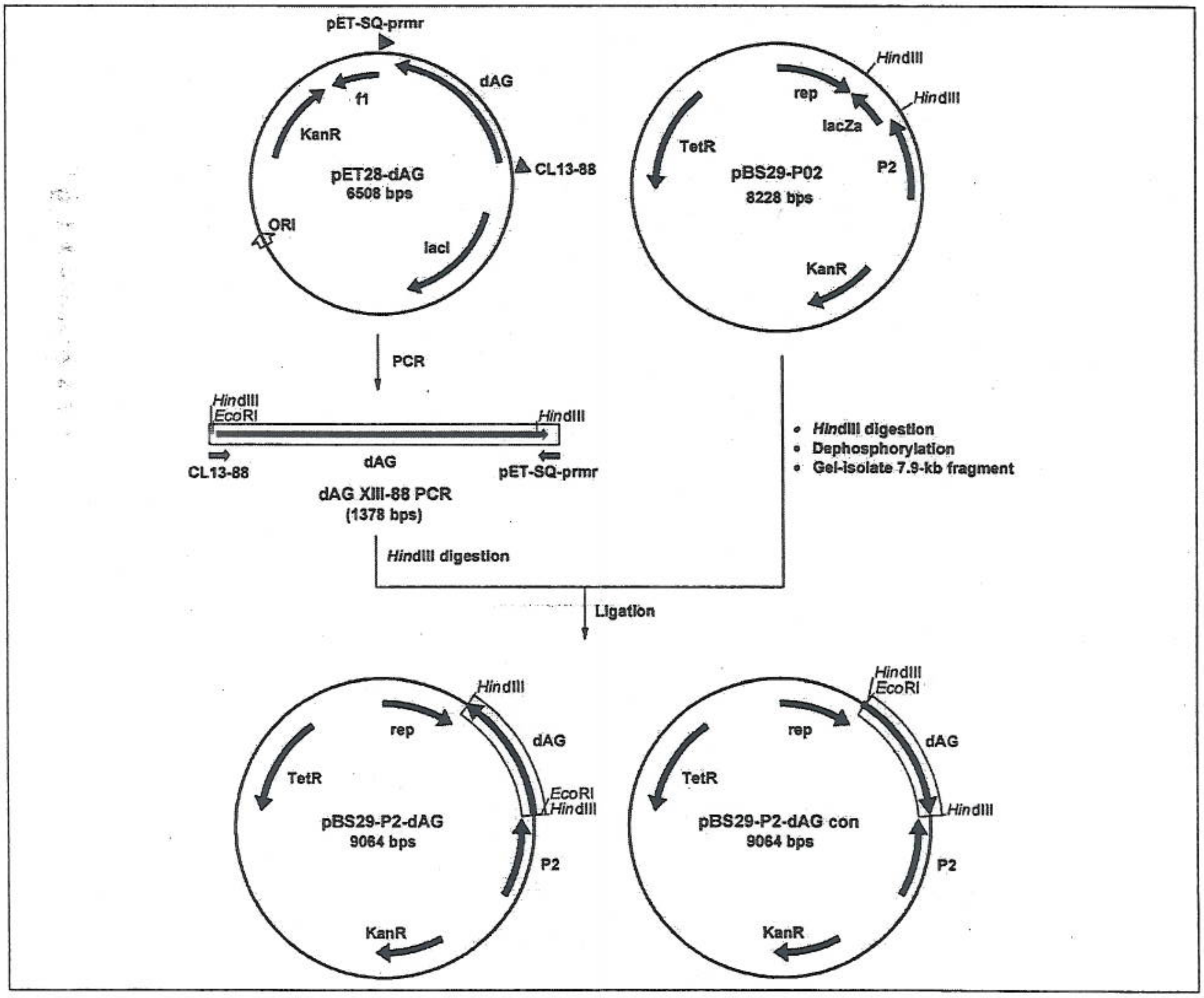
pET-SQ-prmr
dAG
f1
KanR
CL13-88
pET28-dAG
6508 bps
ORI
lacI
HindIII
HindIII
rep
lacZa
TetR
P2
pBS29-P02
8228 bps
KanR
PCR
HindIII
EcoRI
HindIII
CL13-88
dAG
pET-SQ-prmr
dAG XIII-88 PCR
(1378 bps)
HindIII digestion
• HindIII digestion
• Dephosphorylation
• Gel-isolate 7.9-kb fragment
Ligation
HindIII
rep
dAG
TetR
EcoRI
HindIII
pBS29-P2-dAG
9064 bps
P2
KanR
HindIII
EcoRI
rep
dAG
TetR
HindIII
pBS29-P2-dAG con
9064 bps
P2
KanR

FIGURE 5

Schematic of the construction of pBS29-P2-dAG used for expression of α-galactosidase. Relevant abbreviations: *dAG*, the truncated *Streptomyces coelicolor* α-galactosidase gene coding for active enzyme; *pET-SQ-prmr* and *CL13-88*, PCR primers for dAG; *KanR* and *TetR*, kanamycin- and tetracycline-resistance determinants, respectively; *rep*, gene encoding the replication protein *P2*, promoter P2.

sequence separate the −10 and −35 regions of the promoter [26]. The results of the *in silico* analysis indicate that P2 promoter contains sequences that predict it to function as a σ^{70} dependant promoter.

Testing promoter P2 for expression of α-galactosidase

We proceeded to investigate the ability of promoter P2 to direct the expression of a heterologous gene in *P. chlororaphis*. As alluded to earlier, *P. chlororaphis* is an industrially important organism with a capability to produce mcl-PHA and rhamnolipid [12]. We had previously shown that *P. chlororaphis* could grow on inexpensive, surplus soy molasses, but is incapable of metabolizing the oligosaccharides (i.e. raffinose and stachyose) found in the feedstock [27]. If an α-galactosidase gene is cloned and expressed in *P. chlororaphis*, it should promote a more efficient utilization of the carbon sources in soy molasses. Kondoh *et al.* [14] isolated and subsequently constructed a truncated *S. coelicolor* α-galactosidase gene (dAG) containing only the coding sequence of the catalytically active N-terminal domain. A truncated gene is preferred for heterologous gene expression based on our experience because it reduces the possibility of gene modification and inactivation (see e.g. [28]). We PCR-amplified the dAG gene from pET28-dAG using oligonucleotide-primers pET-SQ-prmr (5′-CAGCAGCCAACTCAGCTTC-3′) and CL13-88 (5′-AATTCAAAAGCTTGAATTCGGAGATATACCATGGG-3′), digested the PCR product (1.38 kb) containing the dAG gene with *Hin*dIII restriction enzyme, and subcloned the resultant DNA piece into the 7.9-kb fragment of *Hin*dIII-digested pBS29-P02 vector (Fig. 5). The resultant plasmids, pBS29-P2-dAG and pBS29-P2-dAG con, which contain the dAG gene oriented with and against promoter P2, respectively, were introduced into *P. chlororaphis* by electroporation. The recircularized 7.9-kb fragment of *Hin*dIII-digested pBS29-P02, which is called pBS29-P2(Hd3), was used as a vector-only control plasmid. Cell extracts prepared from the *P. chlororaphis* transformants were assayed for α-galactosidase activity using *p*-nitrophenyl α-D-galactopyranoside as a chromogenic substrate. The results (Table 2) show that the cell extracts of *P. chlororaphis* transformants harboring either pBS29-P2-dAG con or the control pBS29-P2(Hd3) exhibited only a basal level of α-galactosidase activity (2.7–4.5 units/mg protein). The cell-free lysate of pBS29-P2-dAG-transformed *P. chlororaphis*, by contrast, shows an appreciable α-galactosidase activity of 21.4–37.6 units/mg protein.

TABLE 2

Expression of α-galactosidase activity in *P. chlororaphis* transformants

Plasmid in cell	α-Galactosidase activity (units/mg)	
	Trial 1[a]	Trial 2[a]
pBS29-P2(Hd3)	4.5 ± 3.8	3.2 ± 0.6
pBS29-P2-dAG	37.6 ± 5.2	21.4 ± 3.6
pBS29-P2-dAG con	2.7 ± 1.1	2.8 ± 0.6

[a] Each trial was conducted with two separate flasks of culture for each cell type. Two enzymatic assays were carried out with cell extract prepared from each culture. Each value shown thus represents the average of $n = 4$ determinations ± standard deviation.

In conclusion, we have identified a DC3000 sequence, termed promoter P2, located in the chromosomal DNA coordinates 6143993 → 6144829 as having a promoter activity in three important PHA-producing pseudomonads, that is *P. resinovorans*, *P. corrugata* and *P. chlororaphis*, as well as in *E. coli*. Sequence analysis showed that the P2 promoter has all the important features of a promoter belonging to the rpoD17 class. As demonstrated with the expression of an α-galactosidase activity in *P. chlororaphis*, promoter P2 could be useful for expressing heterologous genes in pseudomonads to improve or otherwise modify their metabolic capability. With the successful validation of this strategy to isolate P2 from only ten promoters initially selected, we plan in our future work to screen a larger number of the promoters to isolate additional new sequences for use in expressing heterologous genes in *E. coli* and/or *Pseudomonas*.

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